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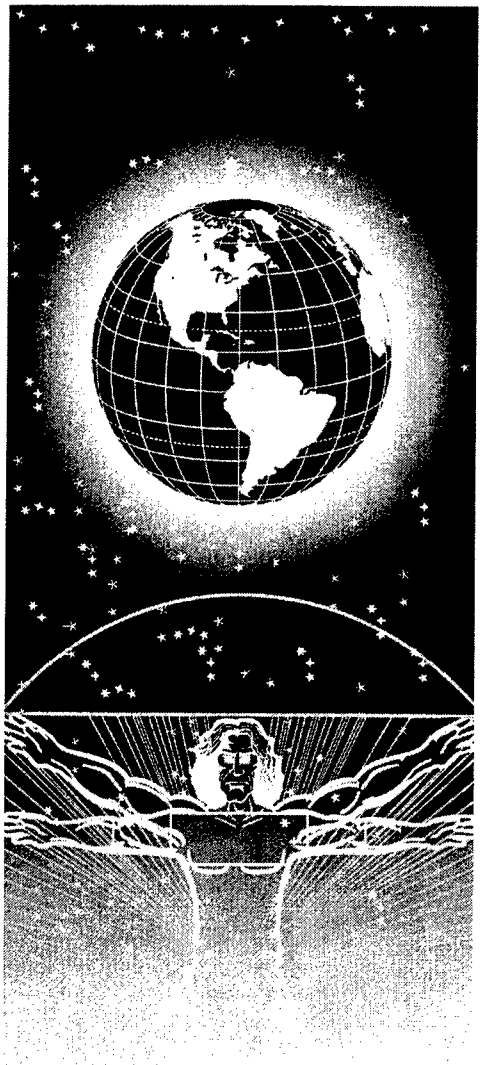

UNITED STATES AIR FORCE RESEARCH LABORATORY

EFFECTS OF JP-8 ON MOLECULAR AND HISTOLOGICAL PARAMETERS RELATED TO ACUTE SKIN IRRITATION

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE DIRECTOR

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13. ABSTRACT *(Maximum 200 words)*

Organic chemicals such as jet fuels and solvents are recognized to cause skin irritation after dermal exposure. The molecular responses to these chemicals that result in acute irritation are not understood well enough to allow the establishment and selection of safe exposure limits. We conducted studies to determine the feasibility of measuring various inflammatory mediators, including IL-1 alpha, inducible nitric oxide synthase (iNOS), and nitric oxide. Male F-344 rats were dermally exposed to JP-8 jet fuel for one hour using Hill Top Chambers. Skin samples were collected at zero, one, two, four and six hours after the beginning of the exposures. Minced skin samples were frozen in liquid nitrogen and pulverized using a stainless steel pulverizer and processed for mRNA and protein analysis. Protein levels were determined using ELISA (IL-1alpha) and western blot (iNOS). Spectrophotometry was used to measure nitric oxide levels in skin using the Griess reagent. Pathological changes in the skin were evaluated histologically, and immunohistochemistry was used to localize IL-1 alpha and iNOS. Northern blot analysis revealed an increase in iNOS mRNA expression by ~25% at 1 hr and ~125% by 6 hr when compared to 0 hr samples. ELISA results for IL-1 alpha in exposed skin samples showed increased levels from 19 to 46% over the 0 hr samples at various time points. Related, iNOS protein levels were also increased by 42 to 130%. The nitrite assay showed that levels decreased during one-hour exposure and then remained relatively constant after the exposure. Immunohistochemical staining indicated increased expression of IL-1 alpha and nitric oxide synthase compared to 0 hr samples.

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INTRODUCTION

Although the skin is generally considered a good barrier to the systemic absorption of chemicals, many chemicals can partially breach the barrier and enter the skin. Toxicity at the site of contact of chemicals is much more common than systemic toxicity due to dermal absorption. According to the U.S. Bureau of Labor Statistics, occupational skin diseases are the second most common types of occupational disease (NIOSH National Occupational Research Agenda Research Areas). In 1994, there were approximately 66,000 reported cases of occupational skin disease, accounting for 13% of all occupational disease. From 1983 to 1994 occupational skin disease increased by 26%, and 75% of workers with occupational skin disease develop chronic skin disease. Occupational skin diseases are believed to be severely underreported and the true rate may be many fold higher. Estimated total annual costs (including lost workdays and loss of productivity associated with occupational skin diseases) may reach $1 billion annually (NIOSH National Occupational Research Agenda Research Areas).

Chemical-induced Skin Irritation

Absorption of any chemical into the skin reflects the potential for irritation. The mechanism of chemical-induced irritation is not completely understood, but the chemical must enter the skin to cause irritation. Chemicals may cause irritation by a nonspecific structural effect on lipids of the skin or by a direct toxic action on the living cells of the skin (Bowman, 1996). Patrick and coworkers (1985) suggested different mechanisms of irritation for different chemicals in their study because blood flow, vascular permeability, skin thickness and infiltration of white blood cells showed a differential response depending on the irritant. They also clearly showed that the same amount of irritants in different vehicles caused different degrees of irritation, presumably by changing the concentration of the irritant that was absorbed into the skin.

Interleukin-1 alpha (IL-1 alpha) has been associated with skin irritation caused by tributyltin (Corsini et al., 1996). These authors found that within 2 hours of exposure to tributyltin, there was a dose-related production of IL-1alpha along with ear swelling and water accumulation.

JP-8 and Skin Irritation

JP-8, when tested on rabbits and guinea pigs, was slightly irritating to the skin and a weak skin sensitizer (Kinkead et al.,1992). JP-8 is more irritating to rats than the JP-4 it replaced (Baker et al., 1999). In general, related petroleum middle distillates cause chronic irritation and inflammation with repeated applications (Grasso et al., 1988; Freeman et al., 1990). Petroleum middle distillates have also been shown to increase the incidence of skin cancer in mice treated for 24 months to a lifetime (Freeman et al., 1993; Broddle et al., 1996). Nessel and coworkers (1999) suggest that prolonged irritation is necessary for tumor formation. There is not enough information available on skin absorption to be able to determine the duration and mass of dermal exposure that would cause irritation.

Theory and Hypothesis

Some recognized mechanisms of irritation are disruption of lipids and membranes, protein denaturation, keratolysis, and cytotoxicity (Elsner, 1994). Fuel components can penetrate the stratum corneum quickly and damage the viable epidermis and the dermis. An effect such as disruption of lipids would involve the release of cellular mediators initiating a complicated biomolecular cascade resulting in edema and erythema. Endogenous modulation of irritant responses is an area that is not understood in a quantifiable manner (Berardesca and Distante, 1994). This toxicity is visible to the histologist, and often by the naked eye, as edema (swelling) and erythema (redness). A simplification of the process is shown in Figure 1.

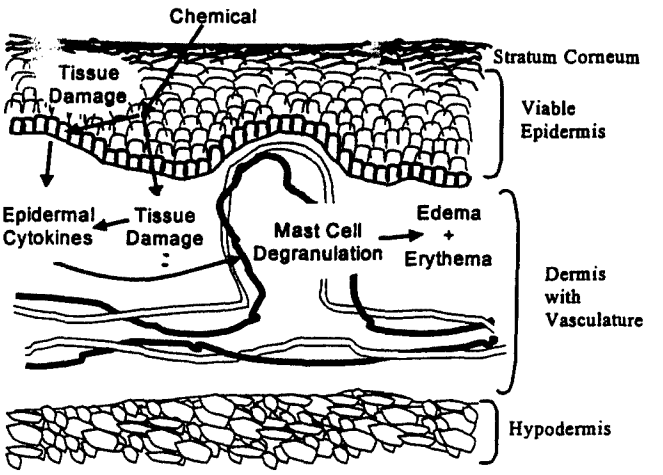

Figure 1. Simple schematic of some of the anatomy and biochemistry of the skin involved in irritation. The irritant chemical must diffuse to the epidermis and dermis where they cause necrosis, apoptosis and adhesion that result in edema and erythema through a complicated cellular response.

The cellular response to irritation is complex and not completely understood. In general, the response is recognized as cellular damage resulting in release of cytokines and chemokines that bring about a complicated vascular response commonly seen on the surface as edema and erythema (Figure 2). Although the specifics of the cellular response continue to evolve with our understanding, the actions of several cytokines and chemokines in irritant dermatitis are understood.

Cytokines are cell signaling molecules that are involved in the inflammatory response. These include the interleukins such as IL-1, IL-6, and other factors such as tumor necrosis factor alpha (Änggård, 1994; Ormerod et al., 1997). IL-1alpha is the major species present in keratinocytes and IL-1β is produced mainly in macrophages (Kupper et al., 1986). Fischer (1995) and coworkers showed that the epidermis of mouse skin has most of the IL-1alpha that is found in the whole skin.

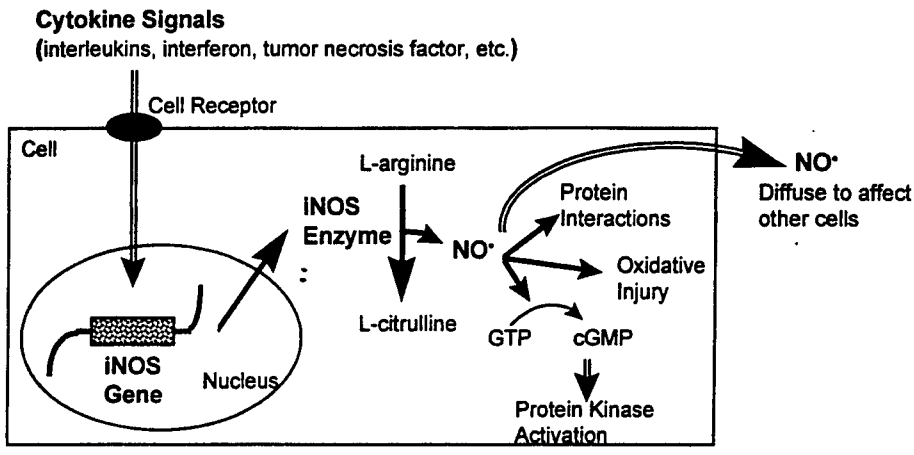

Figure 2. Diagrammatic overview of some of the events that are associated with the upregulation of iNOS during an inflammatory response (based on Änggård, 1994; Vallance and Collier, 1994).

Nitric oxide (NO), a chemokine, also plays an important role in the inflammatory response by causing dilation of the vasculature (Vallance and Collier, 1994). NO is produced from the amino acid L-arginine by nitric oxide synthase (NOS) in macrophages and mast cells in response to the interleukins. NO has been implicated as being a pro-inflammatory chemical and contributes to tissue erythema (Leslie et al., 1994).

Inducible nitric oxide synthase (iNOS) is the inducible, rather than the constitutive, isoform of the nitric oxide synthase enzymes. The iNOS gene is expressed in a number of cell types including smooth muscle cells, fibroblast, keratinocytes, endothelial cells, macrophages, and mast cells (Murphy, 1999; Änggård, 1994). The iNOS gene is upregulated in response to various cytokines, which are involved in inflammatory responses in contact dermatitis (Figure 2, Ormerod et al., 1997). The analysis of iNOS mRNA or protein, as well as the detection of NO or nitrate has been used to study the process of dermal irritation (Ormerod et al., 1997).

The cytokines and chemokines and associated proteins and gene products can be detected at the cellular level before overt skin irritation is observable, and provide an opportunity to measure the early responses to chemical injury (Nickoloff and Naidu, 1994; Casillas et al., 1997).

Purpose of this Study

Ultimately, our goal is to gain a quantifiable, mechanistic understanding of the time course of the skin's response to contact with this fuel, so that assessments can be made about safe exposure scenarios. To develop a mechanistic rat model for human exposure to JP-8, we need to study the time course of cellular and histological responses to JP-8. The purpose of this study is to determine if changes in molecular parameters and histology could be found after a one-hour exposure to JP-8.

MATERIALS AND METHODS

Laboratory Animals and Exposures

Rats were selected for this investigation because of the experience and historical database of the species in dermal absorption studies (both *in vitro* and *in vivo*) in this laboratory (AFRL/HEST). Twenty-five male Fischer rats (CDF® (F-344)/CrlBR, Charles River Laboratories, Raleigh, NC) weighing between 250 and 350 grams were selected as the experimental species (5 rats per each time point). Serology and pathology evaluations indicated the animals were healthy and free of diseases. The animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (NRC, National Academy Press, 1996). The rats were housed one per cage in plastic shoe-box cages and provided a 12-hr light/dark cycle. Temperatures were maintained between 18° and 26°C, and relative humidity was maintained between 30 and 70%. Food (Formulab Rodent Diet, PMI Feeds, Inc., St. Louis, MO) and water were provided *ad libitum*. On the study day, rats were anesthetized with isoflurane (1-chloro 2,2,2-trifluoroethyl difluoromethyl ether) using a vaporizer (Ohio Medical Products). The back of the animal was closely clipped of fur with Oster® animal clippers (McMinnville, TN) and a number 40 blade, taking care not to damage the skin. An Oster® finishing clipper (0.22mm) was used to carefully remove the fur stubble (Jepson and McDougal, 1997). A template equivalent to the diameter of the outside edge of the Hill Top Chamber® was used to mark 4-5 circles with a waterproof marker on the dorsal midline. A Hill Top Chamber® containing 0.250 ml of JP-8 solution was placed on each skin site and the Chamber® was secured with elastic adhesive bandage (Elastoplast® Belesodrof, Inc., Norwalk, CT). Rats were allowed to recover from anesthesia.

Skin Isolation and Preparation

After one hour, the Hill Top Chamber® was removed and excess solution on the exposed area was wiped with cotton gauze. Zero, one, two, four, and six hours after the beginning of the exposure, animals were euthanized with CO_2. Skin samples were collected from the dorsal surface at the level of the scapula at the subcutis. A single skin sample was fixed with 10%

neutral buffered formalin, routinely processed into paraffin embedded sections and prepared for histological or immunohistochemical staining. Two of the remaining skin samples were processed for mRNA analysis, and two samples were used for protein and NO analysis.

Enzyme Linked Immunosorbent Assay (ELISA) to detect IL-1 alpha

The capture antibodies and biotinylated detecting antibodies (Endogen® Woburn, MA 01801) were used according to the manufacturer's instructions in a "sandwich" ELISA procedure, reported previously (Orencole et al., 1997). The limit of detection for IL-1 alpha was 15 - 2000 pg/ml. The IL-1 alpha concentration of each skin supernatant sample (1:5 diluted with Tris buffer) was assayed in duplicate. Additionally, protein determination in skin supernatant was performed using the BCA Protein Assay Kit (Pierce, Rockford, IL) method. Results were read spectrophotometrically at a wavelength of 562nm in a THERMOmax Microplate Reader (Molecular Devices, Sunnyvale, CA). Finally, the data were normalized per mg protein, and expressed as a percent change from 0 hr for each group (mean ± SEM).

Western Blot Analysis for iNOS Level

Proteins (50 μg/lane) from skin samples were denatured at 90°C for 5 min and were resolved on 8% SDS-PAGE according to the procedure of Laemmalli (1970). Following separation, the proteins were transferred to a nitrocellulose membrane according to the method of Towbin et al (1979). The membrane was then blocked with 5% blotting grade blocker and probed with iNOS polyclonal antibody (1:1000; Affinity Bioreagents, Inc. Golden, CO). Following washout with phosphate-buffered saline (PBS), membranes were incubated with peroxidase conjugated secondary antibody and washed three times with PBS. The iNOS band was visualized using enhanced chemiluminescence and autoradiography. iNOS was quantified by comparing band intensities and expressed as a percent change from 0 hr for each group (mean ± SEM).

Isolation and Analysis of mRNA

After the skin was harvested, it was immediately placed into 10mM vanadium ribonucleoside solution (Life Technologies, Rockville, MD) for 30-60 seconds. The sample was removed and placed between two clean metal combs, held together with locking pliers. A scalpel was then passed between the teeth of the comb to cut the skin into strips. The skin was rotated 90° and cut again into small squares. Immediately after mincing, the skin pieces were placed onto a piece of aluminum foil and flash-frozen in liquid nitrogen.

Three of the frozen skin pieces (~100 mg) were then placed into a Bessman steel tissue pulverizer (Fisher Scientific, Cat # 08-418-2) that was pre-chilled in liquid nitrogen. Using a lead hammer, repeated blows to the pestle pulverized the frozen sample. The pulverized sample was immediately removed and placed into a tube containing the RNA isolation reagent (TriReagent, Molecular Research Center, Inc., Cincinnati, OH). The skin was homogenized in the TriReagent using a Tissue Tearor™ electric homogenizer (Biospec Products, Inc., Bartlesville, OK). The RNA isolation procedure was carried out according to the manufacturer's instructions using bromochloropropane for the phase separation and isopropanol for RNA precipitation. The RNA pellet was washed in 75% ethanol and centrifuged. The final RNA pellet was then resuspended in FORMAzol® (Molecular Research Center, Inc., Cincinnati, OH) and stored at –20°C.

To quantify the amount of total RNA isolated, the sample was diluted 1:100 in nuclease-free water and the optical density (OD) measured in a Beckman DU-650 spectrophotometer at 260 and 280nm. The following calculation was used to determine the μg RNA/ml:

$$\mu g\ RNA/ml = (OD_{260})(40\mu g/ml)(\text{Dilution factor [100]})$$

The ratio between the 260nm and 280nm optical densities was used to determine the purity of the isolated RNA. We obtained total RNA with an average yield of 1.0 – 5.0 μg/ml having an OD_{260}:OD_{280} ratio of 1.6 to 1.8.

Northern Blot Analysis of mRNA

Total RNA samples were loaded (10μg/well) onto 1.2% agarose/5.4% formaldehyde gels. Gels were electrophoresed at 70V for 2.5h. After electrophoresis, RNA was visualized with ethidium bromide to determine the quality of the RNA samples. The RNA in the gels was transferred overnight to nylon membranes. Following transfer, RNA was UV-crosslinked in a Stratalinker (Stratagene, La Jolla, CA) set to 1800/Auto-crosslink.

The iNOS (Alexis Corporation, San Diego, CA) and 18S cDNA probes (DECAprime probe from Ambion, Austin, TX) were labeled with [32]P-dCTP by nick translation (Boehringer Mannheim). RNA blots were incubated with the labeled iNOS probe in either PerfectHyb (Sigma, St. Louis, MO) or SuperHyb (MRC, Cincinnati, OH) solution at 55°C for 16hr. After hybridization, blots were washed three times with 0.5X SSC/0.1% SDS at 55°C for 10 min . Blots were then wrapped in cellophane and placed in a film cassette with x-ray film for 1-5 days. After exposure and development, the autoradiographs were scanned with an AGFA DOUSCAN scanner and densitometric analysis performed with Image Gauge version 3.3 software. The blots were then stripped following three to four washes in 0.2X SSC/0.1% SDS at 65°C and hybridized with the 18S cDNA probe. Following overnight hybridization at 68°C and high stringent washes, the blots were exposed to X-ray film for 15-30 min. The ratio of measured band intensities for iNOS were normalized to 18S levels. The expression of iNOS was determined as a mean percentage (± SEM) of 0 hr samples.

Determination of Nitric Oxide Production

Determination of nitric oxide production was achieved by measuring nitrite accumulation in the supernatant solution. Nitrite (NO_2^-) was determined by mixing 100 μl of supernatant and 100 μl Griess reagent (Klebanoff, 1993). The reaction was allowed to proceed for 15 min at room temperature (RT) and the absorbance at 550nm measured. The concentration of NO_2^- was determined from a standard curve of authentic sodium nitrite, treated in a similar manner.

Immunohistochemical Staining of IL-1 alpha and iNOS

Deparaffinized and rehydrated sections were subjected to immunohistochemical staining for IL-1 alpha and iNOS. An avidin-biotin-peroxidase complex method was used according to the procedure included in the Vectastain *elite* ABC peroxidase kit (Vector Laboratory, Burlingame, CA). Briefly, sections were washed with distilled water and incubated with 0.1% Triton X-100 in 0.1% sodium citrate for 30 min at RT prior to quenching of endogenous peroxidase activity with 0.3% H_2O_2. Sections were washed in PBS, and incubated with 5% normal goat serum in PBS for 30 min at RT. Skin sections were incubated either with rabbit anti-rat IL-1 alpha (1:100) or with rabbit anti-rat iNOS (1:100), for 30 min at room temperature. Following PBS washing, the sections were incubated with secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:500) in PBS for 30 min at RT. Peroxidase was detected by the addition of 0.025% diaminobenzidine tetrahydrochloride (DAB; DAKO Corporation, Carpinteria, CA). Finally, all slides were lightly counterstained with hematoxylin, dehydrated and mounted with Paramount® mounting media. The appropriate controls were included during each run to rule out non-specific staining.

Histopathology

When harvesting the skin tissues we avoided aseptic surgical scrubs, which might alter or obscure key diagnostic features. Biopsies were elliptical in shape with the long axis oriented parallel to the direction of hair growth. This provides a longitudinal microscopic view of hair follicle structures rather than undesirable cross sectional perspectives. Skin specimens were flattened on a piece of paperboard or photographic paper and gently stretched before immersion in 10% neutral buffered formalin for 24 hours. Following fixation, skin samples were processed using Standard Operating Procedures for paraffin processing, embedding, and sectioning. Hematoxylin and eosin stained slides were assessed in random order by group and scored as described previously (McDougal et al., 1997). The scoring guidelines were: 0= < 3 extravascular dermal granulocytes; 1= 3 or more extravascular dermal granulocytes, variable degree of basement membrane separation, epithelial swelling, vacuolation, and spongiosis; 2 = 1+ epidermal transmigration of granulocytes.

RESULTS

Effects of JP-8 on IL-1 alpha Levels

Skin supernatant solution processed from skin samples at different time points was utilized for IL-1 alpha estimation. As shown in Figure 3, JP-8 exposed cytokine IL-1 alpha was significantly increased from 19 to 46% compared to the 0 hr samples. Maximum IL-1 alpha activity was seen at one hour post exposure. These results indicate that IL-1 alpha respond rapidly to JP-8 within one hour after initiation of JP-8 exposure. Early activation of IL-1 alpha occurred in JP-8 exposures.

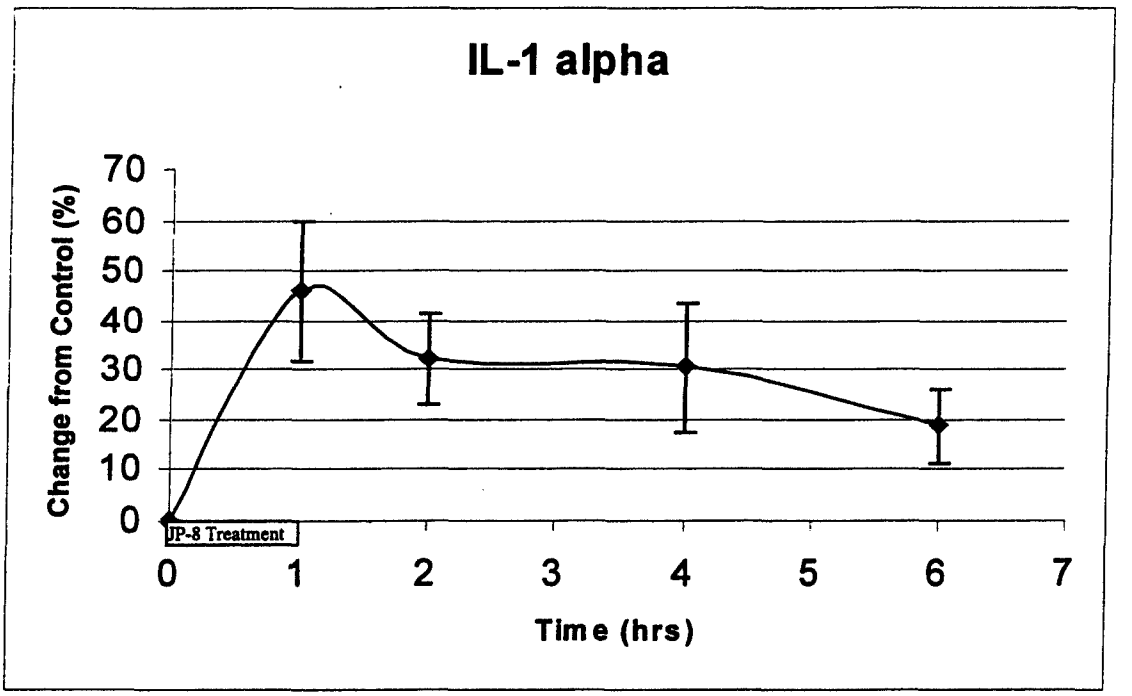

Figure 3. ELISA results showed that interleukin-1 alpha rapidly increased during the one-hour exposure to JP-8 and slowly decreased by 6 hours. Error bars depict standard error of the mean (n = 5 at each time point).

Effects of JP-8 on iNOS Levels

Western blot analysis revealed that iNOS induction was involved in the skin after JP-8 exposure. Figure 4 shows that the iNOS expression level was increased by 42 to 130% compared to 0 hr samples. The significant induction of iNOS was observable at 4 hour post exposure of JP-8 indicating the activation of iNOS occurred after IL-1 alpha induction at one hour and then increased over a 4 hour period.

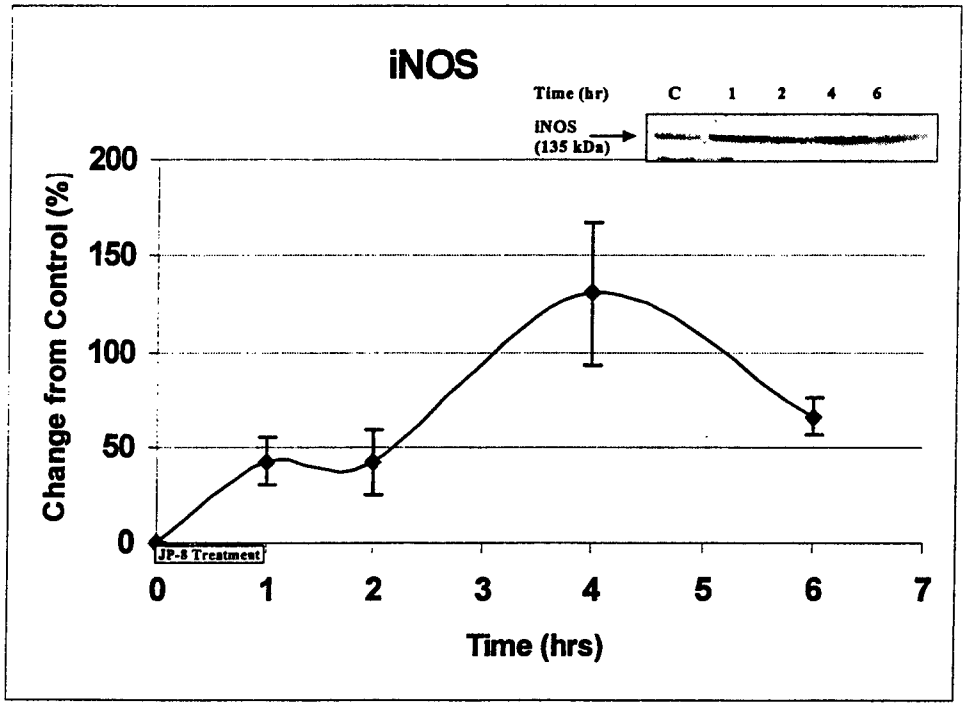

Figure 4. Western blot analysis revealed that inducible nitric oxide synthase expression increased due to JP-8 exposure, and it was maximum at 4 hours after the beginning of the exposure. Error bars depict standard error of the mean (n=5 at each time point).

Effects of JP-8 on iNOS m-RNA Levels

Northern blot analysis of total RNA isolated from rat skin was performed to characterize expression of iNOS following exposure to JP-8 jet fuel (Figure 5). Densitometric analysis of band intensities compared the percent change of iNOS mRNA expression from 0 to 6 hr post-exposure to JP-8. At 1 hr post-exposure to JP-8, iNOS mRNA expression increased by ~25% above 0 hr samples. The expression of iNOS mRNA steadily increased through 6 hr with an observed maximum of ~125%.

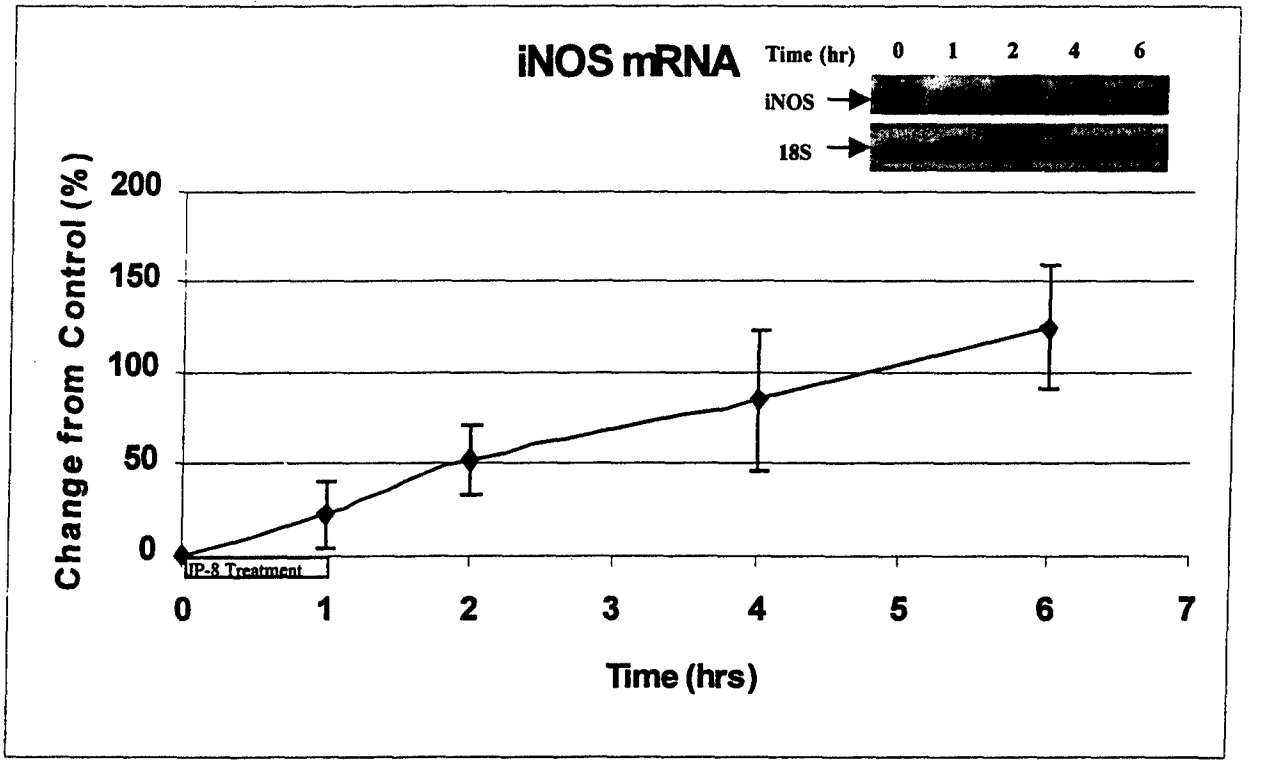

Figure 5. Northern blot analysis revealed that inducible nitric oxide synthase mRNA expression increased due to JP-8 exposure, and it was maximum at 6 hours after the beginning of the exposure. Error bars depict standard error of the mean (n=5 at each time point).

Effects of JP-8 on Nitric Oxide

The production of nitric oxide as a result of JP-8 exposure was determined by estimating the levels of nitrite (NO_2^-) through Griess reagents in the skin supernatant solution. Figure 6 shows that detectable nitrite levels decreased by one hour and then remained relatively constant for 6 hours.

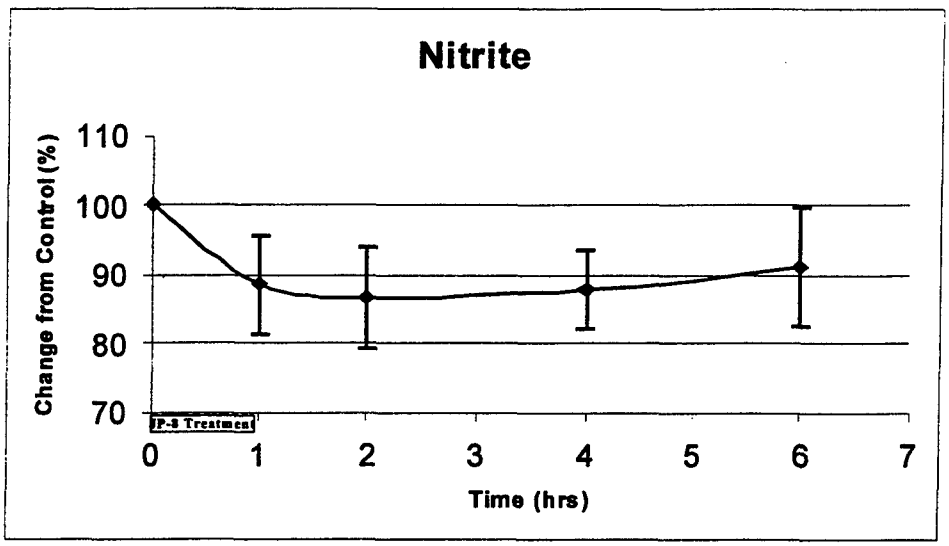

Figure 6. Nitrite levels were decreased during the one-hour exposure to JP-8 and remained relatively constant for five hours after the end of the exposure. Error bars depict standard error of the mean (n = 5 at each time point).

JP-8 Activation of IL-1 alpha and iNOS by Immunohistochemical Staining

In order to confirm the molecular biomarkers of dermal irritation and inflammatory response to JP-8 in rats, immunohistochemical staining of skin sections for IL-1 alpha and iNOS showed increased immunolocalization compared to 0 hr samples. Both IL-1 alpha and iNOS positive staining were primarily localized in the epidermis of the skin sections (Figure 7 (A, B, C, & D).

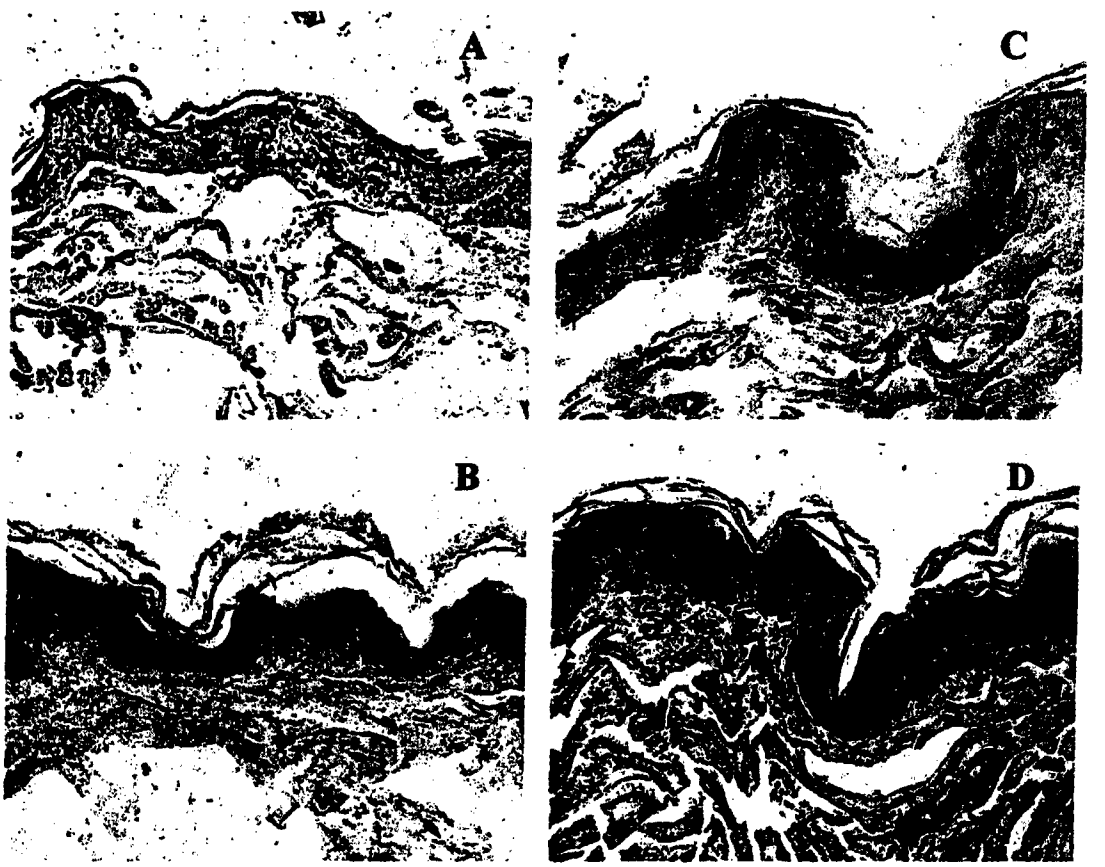

Figure 7. Skin section (X 40) treated one hour with JP-8 shows higher immunohistochemical staining of interleukin-1 alpha (B) and inducible nitric oxide synthase (D) in the epidermis compared to 0 hr samples (A and C, respectively). Skin sections are taken three hours after the end of exposure.

Histopathology

Mean histopathologic assessment scores are presented in Table 1 below. Increased histopathologic assessment scores were observed from two, four and six hours time points. These results indicate that earliest JP-8 induced histopathological change, (granulocyte emigration), can be seen as early as two hours and changes were most prominent by 6 hours after the start of exposure.

Table 1. Mean Histopathologic Assessment Scores for one-hour JP-8 Exposure

Time (Hours)	Mean Scores* (range)
0	0 (0-0)
1	0 (0-0)
2	0.4 (0-1)
4	1.0 (0-2)
6	1.6 (1-2)

*Scoring guidelines based on the following criteria: 0= < 3 extravascular dermal granulocytes; 1= 3 or more extravascular dermal granulocytes, variable degree of basement membrane separation, epithelial swelling, vacuolation, and spongiosis; 2 = 1+ epidermal transmigration of granulocytes. n=5 at each time point.

DISCUSSION

We have measured molecular responses in the skin following a one-hour exposure to JP-8 on the skin surface and have seen changes in several parameters that appear to represent an acute irritant reaction. We observed these changes at earlier times than previously reported. These changes appear to be part of an "irritant cascade" that occurs rapidly after dermal exposure to some chemicals. This response to injury, which ultimately results in inflammation, is complex. Figure 8 shows the parameters that we measured, which we suspect are involved in the irritant response.

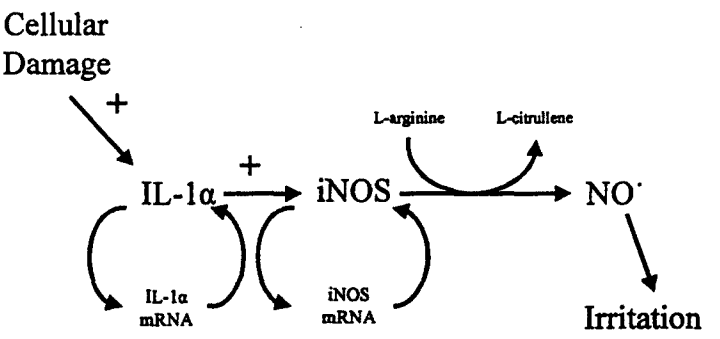

Figure 8. Proposed irritant cascade simplified to include parameters we measured.

Levels of IL-1alpha were increased by about 50% one hour after the initiation of the JP-8 exposure and slowly decreased over the next 5 hours (Figure 3). The one-hour value is probably not the real peak. It is just as likely that the levels peaked prior to one hour and our first time point caught them on the way down. It is also possible that the peak is between one and two hours. This rapid response of IL-1 alpha is surprising if it represents new levels of protein being transcribed and translated in such a short period. JP-8 would have to diffuse through the stratum corneum and into the epidermis to initiate the transcription and translation. Several authors (Kupper, 1990; Corsini and Galli, 1998; Murphy et al., 2000) suggest that there is a shield or

umbrella of IL-1 alpha in the stratum corneum and epidermis that can start the complicated sequence of inflammatory actions in the dermis when released. It is unlikely that the one-hour level represents the release of this constitutive IL-1 alpha, because the homogenization technique should allow us to detect both intracellular and extracellular IL-1 alpha. Therefore, if constitutive IL-1 alpha was just released and not manufactured, the protein level should not change at one hour. If our homogenization technique is not sufficient to completely lyse the skin cells, it is possible that JP-8 could have an effect on the cell membrane fluidity. This effect would make JP-8 exposed cells easier to homogenize, thus releasing more IL-1 alpha. Therefore, it is unclear whether or not we are measuring newly synthesized protein. IL-1 alpha mRNA levels and JP-8 exposures with sampling at times shorter than an hour will help clarify our results.

Our detection of changes in IL-1 alpha levels is earlier than previously reported. Corsini and coworkers (1996) showed that tributyltin-induced skin irritation to mouse ear is related to IL-1 alpha increases intracellularly and extracellularly at four hours. They found about a 50% increase in both the IL-1 alpha content of ear slices and the media after overnight incubation. They didn't look for changes at earlier time points. Ear swelling and water content were also greater at 4 hours than at 1 hour or 12 hours. They were not able to detect any changes in IL-1 alpha mRNA using RT-PCR. In another study, IL-1 alpha release from human skin equivalent cultures (Perkins et al., 1999) after 20 or 24 hour treatments with consumer products correlated well with human irritancy testing. They didn't report release at earlier time points.

Immunohistochemistry of IL-1 alpha showed that there was an increased density of antibody staining in the epidermis after one hour of exposure. This change persisted at all later time points. However, there was not a further increase in density detectable by the naked eye at later time points. IL-1 alpha is most prevalent in keratinocytes but also present in Langerhans cells and melanocytes (Kupper, 1990). Several authors (Kupper, 1990; Corsini and Galli, 1998; Murphy et al., 2000) suggest that there is a shield or umbrella of IL-1 alpha in the stratum corneum and epidermis which can start a complicated sequence of inflammatory actions in the dermis when released. Wood and coworkers (1996) found by immunohistochemistry methods that IL-1 alpha increased in the mouse epidermis and dermis 10 minutes after the skin barrier

was disrupted by tape stripping. They also found that the levels were still elevated at 2 and 4 hours but that they returned to normal by 24 hours. This study suggests that we should also find IL-1 alpha elevated when we look at times earlier than one hour into the exposure.

Nitric oxide produced by inducible nitric oxide synthase (iNOS) from L-arginine has been implicated in the inflammatory response (Ormerod et al., 1997) as the endothelium-derived vascular relaxation factor (Henry et al., 1993). iNOS was originally recognized to be in macrophages, but more recently has been identified in keratinocytes (Arany et al., 1996) where it's role in wound repair has been recognized (Frank et al., 1999; 2000). In our studies, JP-8 caused the levels of iNOS in the whole skin to increase by more than 100% during the first 4 hours and decrease again over the next 2 hours (Figure 4). This seems to be a reasonable time frame for induction of new protein. iNOS mRNA levels (Figure 5) increased linearly and levels doubled by 6 hours after the beginning of the exposure. iNOS protein levels appeared to drop after 4 hours although mRNA levels were constant or increasing. This relationship suggests that there is more to regulating iNOS protein levels than what is seen at the transcriptional level.

Nitrite (NO^-_2) and nitrate (NO^-_3) are stable breakdown products of NO (Vallance and Collier, 1994). Total nitrite levels (Figure 6), as an indication of the amount of NO formed, dipped slightly during the one-hour exposure and then remained unchanged. We would have expected that the levels of the stable products of NO would increase with a corresponding increase in iNOS. Because our method only measured nitrite not nitrate, we might see a different picture when the nitrate is reduced to nitrite prior to running the Griess reaction. Another possibility for the lack of changes is that some of the NO may have formed peroxynitrite (Bartosz, 1996) or other reaction products and thus not be part of the stable nitrate pool. Determination of iNOS activity (L-citrulline production from L-arginine) may clarify this discrepancy.

Immunohistochemistry of iNOS showed a pattern similar to IL-1 alpha – increased staining at one hour, but exhibited no clear differences with time up to 5 hours after the end of the JP-8 exposure. Becherel and coworkers (1997) found with immunohistochemistry that human keratinocytes near the dermoepidermal junction expressed iNOS in response to IgE/allergen

immune complexes. Noninflamed skin did not show iNOS immunostaining. There is no clear information about the time course of iNOS induction in the literature.

Traditional histology showed that we are able to detect extravascular granulocytes in rats within one hour after the end of a one-hour exposure. This is the time when the IL-1 alpha protein levels were highest and IL1-alpha is recognized to be a mediator in this inflammatory response (Ormerod et al., 1997). This is similar to what we previously found in rats with dichlorobenzene (McDougal et al., 1997). We were unable to detect histological changes earlier than one hour after the exposure; therefore, the biological responses that we have identified provide earlier markers than obtained with histology.

SUMMARY

Our initial efforts at detecting early molecular events in the irritant and inflammatory response have been successful. We see coordinated changes in a signaling cascade that have not previously been identified from chemical irritation or damage. Refinement of our methodology and expansion of the parameters of our investigation into such things as half-lives of mRNA and the cytokines in the skin, provide hope that this process can be understood well enough to be predictive.

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